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Activity in varicosities within the myenteric plexus between and during the colonic migrating motor complex in the isolated murine large intestine

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Abstract

Background—Neuronal communication within the myenteric plexus occurs when action potentials along nerve fibers produce Ca^{2+} transients in varicosities leading to exocytosis of vesicles and neurotransmitters release. We used Ca^{2+} transients in varicosities to monitor action potential activity in myenteric nerve pathways both between and during the colonic migrating motor complex (CMMC) in the isolated murine colon.

Methods—Strips of longitudinal muscle were removed to reveal the myenteric ganglia, which were then loaded with Fluo-4.

Key Results—Many varicosities, including synaptotagmin 1 labeled varicosities, exhibited ongoing Ca^{2+} transients (duration of unitary Ca^{2+} transient 3.9s). Between CMMCs, varicosities fired at a frequency of 0.6 Hz, which correlated with spontaneous inhibitory junction potentials in the circular muscle, suggesting they were mainly in inhibitory nerve pathways. During a CMMC other previously quiescent varicosities fired at 1.3 Hz (max 2.0 Hz) for the duration (24 s) of the CMMC, suggesting they were on excitatory nerve pathways. Activity in varicosities was correlated with Ca^{2+} transient responses in a number of neurons. Some varicosities appeared to release an inhibitory neurotransmitter that reduced activity in nNOS-positive neurons. Varicosities along the same nerve fiber exhibited identical patterns of activity that allowed nerve fibers to be traced throughout the myenteric plexus and intermodal strands. Activity in varicosities was reduced by hexamethonium (100 μM), and blocked by ω -conotoxin GVIA (200 nM) and tetrodotoxin (1 μM ; TTX).

Conclusions & Inferences— Ca^{2+} imaging of varicosities allows for a determination of activity in neural pathways within the enteric nervous system.

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Keywords

Calcium imaging; CMMC; colon; large intestine; myenteric neurons; varicosity

INTRODUCTION

The very accessible enteric nervous system (ENS) is found in the GI tract of mammals and uses many of the neurotransmitters found in the mammalian spinal cord and brain.¹ The ENS consists of two neural networks of interconnected ganglia called the myenteric plexus and submucous plexus that primarily regulate intestinal motility and secretion, respectively.² The myenteric plexus forms an almost two dimensional sheet lying between the longitudinal and circular smooth muscle layers. Each myenteric ganglia can contain many different functional classes of neurons, including sensory neurons, excitatory and inhibitory motor neurons, as well as ascending and descending interneuron's that communicate between myenteric ganglia and with submucosal ganglia.^{2,3} Even closely apposed myenteric neurons within a single myenteric ganglion can show very different activities,^{4,5} presumably because they are insulated from each other by glial cells.⁶ Despite this complexity, myenteric neurons appear to be largely arranged in polarized ascending excitatory and descending inhibitory reflex pathways.^{7,8}

There have been a number of attempts to determine the characteristic firing patterns in myenteric neurons using extracellular recording during spontaneous and stretch evoked activity in order to determine how the network generates and integrates neuronal activity.^{9,10,11} A potential problem with extracellular recording is that the electrodes themselves may mechanically distort the soma of neurons and cause an artificial discharge. To avoid this problem intracellular microelectrodes have been used to record from single myenteric neurons during reflex activity.^{12–16} Such recordings can monitor the synaptic responses in myenteric neurons and have provided important information, they are both difficult and time consuming, and it can be difficult to fit the characteristic discharge of single neurons into a viable nerve circuit. There have been recent attempts to record from large number of myenteric neurons and their effector cells using Ca^{2+} imaging¹⁷ and voltage sensitive dyes¹⁸ in more or less intact preparations. We have also used a Ca^{2+} imaging approach to study the activity in many neurons and their effector cells (i.e. ICC-MY, glial cells and muscle) at the same time during a stereotypical, rhythmic, neurally generated motor pattern called the colonic migrating motor complex (CMMC) in the large bowel.^{4,5,6,19} Our aims have been to understand the activation of multiple neural pathways that underlie this important colonic motor event that propels fecal pellets, at least in the mouse.²⁰

The CMMC, is a complex motor event^{21–24} that appears to be initiated mainly by 5-HT released from enterochromaffin cells in the mucosa.^{4,5,20} The released 5-HT activates the mucosal endings of myenteric and submucosal AH/Type II neurons.^{5,25} An increase in activity of AH neurons appears to be necessary for generating the CMMC that then propagates along the colon because of the interneuronal pathways, which in turn converge onto other AH neurons.^{4,5,26} Once the complex is generated, excitatory motor neurons are

activated and some inhibitory motor neurons decrease their activity to allow for full excitation of myenteric pacemaker cells (referred to as myenteric interstitial cells of Cajal or ICC-MY) that in turn activate the neighboring longitudinal and circular smooth muscles at the same time.^{4,5,19, 27}

Myenteric neurons communicate with each other and their target cells via varicose nerve fibers, which often form close contacts with myenteric neurons.^{28–32} Although it was generally believed that varicosities often released their neurotransmitters some distance from the membranes of target cells, it now appears that at least some varicosities can make specialized junctional contacts with muscle cells in blood vessels,³³ intramuscular interstitial cells of Cajal,³⁴ colonic myenteric pacemaker ICCs and muscle,¹⁹ and glial cells.⁶ Transmitter release from varicosities has been intensely studied in the sympathetic nervous system.^{35–37} These studies have shown that a single action potential, which is about 1–2ms in duration, propagating along a single nerve fiber generates a Ca^{2+} transient (duration 3–4s), in all varicosities along its terminal branches at the same time.^{35–37} An invading action potential depolarizes varicosities to cause Ca^{2+} influx through mainly N-type calcium channels. Importantly, this suggests that Ca^{2+} transients in varicosities are a reporter of an action potential propagating along a nerve fiber.

In this study, by measuring Ca^{2+} transients in varicosities, our aims were to determine the patterns of neuronal firing between and during CMMCs and, importantly, to establish whether such interneuronal communications can be correlated to previously reported functions of myenteric neurons during the CMMC. This would give the possibility of studying both neural input and neuronal responses at the same time, as we have recently demonstrated for ICC-MY.¹⁹

METHODS

Tissue Preparation

Adult C57/BL6 mice of either sex (4–6 weeks of age) were sacrificed by isoflurane inhalation and cervical dislocation in accordance with the requirements of the National Institute of Health, reviewed by the Animal Ethics Committee at the University of Nevada, Reno. Ventral midline sections were made, and the entire colon was removed, and placed in a large Sylgard (Dow- Corning, Midland, MI)- layered dissecting dish containing Krebs-Ringer buffer solution (KRB; constitution in mM: NaCl: 120.35, KCl: 5.9, NaHCO_3 : 15.5, MgCl_2 : 1.2, NaH_2PO_4 : 1.2, dextrose: 11.5, CaCl_2 : 2.5; pH: 7.4; 21°C), bubbled with a physiological air mixture (97% O_2 –3% CO_2).

Colonic preparations were made for both local and distal mucosal stimulation, as described previously.⁵ Briefly, the isolated colon was cut open in the middle (10–15 mm), and strips of longitudinal muscle were peeled away to reveal the myenteric plexus that remained attached to the circular muscle. This dissected middle region was then pinned in a Sylgard-layered recording chamber, serosal side up, using tungsten micropins (Fine Science Tools, Foster City, CA). The distal end was also opened and pinned with the mucosa uppermost for mucosal stimulation. For preparations, in which the effects of local stimulation were studied, a small polyurethane tube was embedded into the Sylgard of the recording chamber, and was

connected to a picospritzer (Picospritzer II, General Valve Corp., Fairfield, NJ), spritzing brief (~40ms) pulses of N₂.⁵ Following dissection, preparations were equilibrated in oxygenated 37°C KRB perfused for approximately 60 minutes.

Fluorescent Dye Loading

Following equilibration, perfusion was stopped, the KRB in the recording chamber was allowed to cool down to room temperature, and the volume of KRB was minimized. Tissues were incubated for 15 minutes with 50 nM Fluo-4 AM (Molecular Probes, Eugene, OR) dissolved in 0.02% dimethyl sulfoxide (DMSO) and 0.01% of the non-toxic detergent Chremophor EL (Sigma-Aldrich, St. Louis, MO). Following dye incubation, the perfusion was again restored, and tissues were allowed to de-esterify in 37°C oxygenated KRB for another 15 minutes.²⁶ In experiments where local stimulation was used to examine the function of myenteric Dogiel Type II neurons, probenecid (500 µM) was added to the perfused solution to prevent dye expulsion.⁵

At the conclusion of some experiments, where the goal was to explore the function of Dogiel Type II neurons, tissues were loaded with Mito Tracker Red CMXRos (Molecular Probes), which preferentially labels these neurons, as previously described.⁵

Image Acquisition

Fluorescent Ca²⁺ signals were visualized on a Nikon Eclipse E600FN upright fluorescent microscope using Nikon Fluor R water immersion objectives (10–100x; Nikon, USA) and a modified GFP dichroic cube (ex: 488 nm, em: 543 nm; Chroma Technology Corp., Bellows Falls, VT). Images (2000–4000 frames at 32.4Hz) were acquired using an Andor iXon +897 EMCCD camera (Andor Technology, Belfast, Northern Ireland) using the Andor Solis 4.14 software running on a Windows-based PC.

Mucosal Stimulations

CMMCs and local reflexes were mechanically evoked as described previously.^{4,5} Briefly, a small (6mm-wide, flat head) paintbrush was used to stroke the mucosa at the distal end of the preparations (3–5 strokes) and generate CMMCs which propagated up the length of the colon, and were visualized in the recording field of view ~7 seconds following stimulation.⁴ Local stimulations consisted of brief (25–40ms) puffs of N₂ (1–5 puffs) delivered by the Picospritzer through a small (~500µm) hole in the polyurethane tube embedded in the Sylgard underneath the site of recording.

Immunohistochemistry

Following Ca²⁺ imaging experiments, shapes of ganglia were revealed by application of 50mM KCl directly into the recording chamber, which activated all neurons, while images were being acquired. Each tissue was trimmed to the region that contained the imaged ganglia. These regions were re-pinned onto small (15ml) Sylgard-layered fixation chambers, and were fixed in 4% paraformaldehyde overnight. Following fixation, tissues were rinsed for several hours with 0.01 M phosphate buffered saline (PBS, pH 7.2), and were then cleared in DMSO (3 x 15 minutes). Tissues were then blocked in 1% bovine serum albumin (BSA), and were incubated for 48 hours in primary antibodies diluted in 0.5% Triton-X

(Sigma-Aldrich, St. Louis, MO) sequentially. Following primary antibody incubation, tissues were washed in 0.01M PBS for up to 24 hours, and were then incubated with secondary antibodies for an hour, in sequence. Tissues were then washed in PBS, and were mounted onto glass microscope slides (Fisher Scientific, Pittsburgh, PA, USA) using Aquamount (VWR International, West Chester, PA, USA).

Primary Antibody	Raised in	Dilution	Source
Synaptotagmin 1	Rabbit	1:1000	Sigma, MI, USA
Calretinin	Goat	1:1500	Abcam, Cambridge, MA, USA
nNOS	Sheep	1:1000	P.C. Emson, UK

Whole mount tissues were imaged using a Zeiss LSM 510 META confocal microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) with 10–100x lenses. LSM stacks were acquired as a stack of grayscale 16-bit tiff files, and were pseudocolored, as necessary to display correlation, by assigning each stack to a an RGB channel in Photoshop CS5 (Adobe Systems Inc., San Jose, CA).

Image Processing and Analysis

Raw Solis.tiff stacks were analyzed on a MacPro desktop computer (Apple Inc., Cupertino, CA) using an in-house analysis software (Volumetry 6a & Volumetry 7, GWH). Movement of tissues in both the x and y direction were tracked, and the stacks were stabilized to allow for Ca^{2+} signals in individual regions of interest (ROIs) to be measured. Fluo-4 signals are reported as average intensity inside an ROI in 8 or 16-bit intensity units. Spatio-temporal maps were constructed by drawing rectangular regions of interest over areas and averaging the pixel intensities perpendicular to the long axis.³⁸ Care was taken, when drawing regions of interest to avoid any background fluorescence, from either muscle cells or other varicosities or neurons, which would bleed through the ROI.

To quantify activity in varicosities in a ganglia, the movie was differentiated ($t = 200$ ms), then spatially (Gaussian blur: 1 sd, 5x5 pixels) and temporally filtered (200 ms average) to remove the majority of noise. A 10 s period (150 frames) was used for analysis. A threshold was set which highlighted all varicosities displaying active Ca^{2+} transients. To identify varicosities that fired one or more Ca^{2+} transients during the analysis period, all frames were summed temporally (maximum sum value possible = 150). The resulting summed frame was duplicated, and modified as necessary (to remove unwanted nerve cell bodies etc) to calculate the total area of active varicosities. Bitmasks were also created for a single fiber or cluster of fibers using a frame in which those fibers were active. Then, for each frame of the movie, any pixels within the bitmask that had intensity values above the set threshold were counted and were expressed as % of total varicosity bitmask area.

Immunohistochemical overlays with Ca^{2+} imaging mean or maximum stack frames were constructed in Adobe Photoshop CS5, by reducing the translucence of the uppermost layer. Schematic and pseudocolored renditions were made in CS5 using the software's layer masking tools.

Statistical Analysis

Results are expressed as a means $\pm$ S.E.M. Student's unpaired t test and one-way ANOVA were used where appropriate. $P < 0.05$ was accepted as statistically significant. The n values represent the number of animals on which observations were made.

Drugs Used

Hexamethonium, nicardipine, ω -conotoxin GVIA and tetrodotoxin (TTX), were all purchased from Sigma, CA, USA. Potassium chloride was purchased from Fisher Scientific (Pittsburgh, PA).

RESULTS

Monitoring activity in nerve fibers in intermodal strands

Calcium imaging of myenteric ganglia revealed activity in many varicosities in nerve fibers throughout the ganglia and in intermodal strands. Ca^{2+} transients in a single nerve varicosity along one of these nerve fibers in an intermodal strand is demonstrated in Figure 1A (** and arrow in upper panel) by a line scan, a differentiated trace and a spatio-temporal map (ST-Map; Figure 1A, I). Fast Ca^{2+} transients in one of these varicosities summed periodically to give an underlying sustained rise in Ca^{2+} (Figure 1A, I). The individual Ca^{2+} transients in varicosities had a fairly constant amplitude and a similar rate of rise as shown by the differentiated trace.

When regions of interest were taken across an entire intermodal strand the differentiated Ca^{2+} transients revealed complex spontaneous activity from a number of nerve fibers and varicosities (Figure 1A, II and III). The activity in nerve fibers is also represented as an ST-Map formed from the same region (Figure 1A, II). The activity from a single varicosity shown in Figure 1AI was extracted from the thin green rectangle in the ST-Map (Figure 1A, I). Interestingly, the two intermodal strands shown in the panels of Figure 1A exhibited different patterns and amplitudes of Ca^{2+} activity at similar overall frequency (0.9Hz-red rectangle and 1.1Hz-blue rectangle; Figure 1A II and III). In a typical experiment, 14 ± 2.0 ($n=7$; at $\times 40$ magnification) nerve fibers could be distinguished in an intermodal strand using Ca^{2+} imaging.

Varicosities lying along the same nerve fiber exhibited identical patterns of Ca^{2+} transients, allowing the nerve fiber to be traced through an intermodal strand as shown in Figure 1B (orange arrows) and the 8 orange traces (Figure 1B, I). The orange traces are summed together in Figure BI. This particular nerve was traced from one intermodal strand through a ganglia and into the adjacent strand (see orange arrows in Figure 1B). In this example, using the same criteria, we were able to trace a further 5 nerve fibers (Figure 1B, arrows); the traces in Figure 1B (I I–vi) show the summed activity from eight varicosities lying along each particular nerve fiber. The summed activity of all the traced nerve fibers in this example is superimposed in the bottom trace (Figure 1B, II), which resembles multiunit electrical recording from a complex nerve bundle (see Discussion).

Single Ca^{2+} transients in varicosities in intermodal strands and in myenteric ganglia had a duration of $3.9 \pm 0.4\text{s}$, a rise time of $0.30 \pm 0.04\text{s}$ and a decay time of $3.6 \pm 0.4\text{s}$ (100 transients; $n=6$). The size of varicosities observed using Ca^{2+} imaging was $2.0 \pm 0.1\text{ }\mu\text{m}$, which was slightly larger than those obtained by immunohistochemical labeling of varicosities with synaptotagmin 1 ($1.4 \pm 0.1\text{ }\mu\text{m}$; see below). Differences may be attributed to tissue shrinkage during fixation.

Monitoring activity in varicosities in myenteric ganglia

The maximum Ca^{2+} intensity throughout the image sequence (Fluo-4 max) revealed numerous varicosities throughout myenteric ganglia and interganglionic spaces (Figure 2A). A sampling of spontaneous Ca^{2+} transients in various varicosities both within and outside a ganglia between CMMCs revealed random patterns of activity that occurred as either a single transient or bursts of Ca^{2+} transients (Figure 2A, B). This apparently random activity in varicosities is summarized in the "bar code", which demonstrates that there was no obvious pattern to this activity (Figure 2A). The complexity of activity in varicosities is further exemplified in Figure 2B, which shows the spontaneous Ca^{2+} transients in 8 varicosities lying over a neuron (N). There was little correlation of varicosity activity with the Ca^{2+} transient activity in this particular neuron.

When the mucosa was stimulated either under the recording site (local stimulation) or by anal mucosal stimulation (see below) the overall frequency of firing in varicosities increased by $0.6 \pm 0.1\text{ Hz}$ (range 0.1 to 1.4 Hz) to $1.3 \pm 0.1\text{ Hz}$ (range 0.7 to 2.0 Hz) for a period of $23.7 \pm 2.0\text{s}$ (range 12.9 to 32.4s; $n=4$) during the evoked CMMC (Figure 2D). The firing frequency in different varicosities between and during the CMMC evoked by nerve stimulation is shown in Figure 3.

Effects of TTX and hexamethonium

Tetrodotoxin ($1\text{ }\mu\text{M}$; $n=4$) blocked all activity in varicosities and neurons; whereas, the nicotinic antagonist hexamethonium ($100\text{ }\mu\text{M}$; $n=4$) abolished 75% (102 varicosities, $n=4$) of the spontaneous Ca^{2+} transients in varicosities (Figure 2B and D).

Synaptotagmin 1 is contained in many, but not all varicosities

Upon depolarization of some varicosities, the influx of Ca^{2+} ions binds to the presynaptic protein synaptotagmin 1 leading to rapid exocytosis of vesicles and neurotransmitter release from varicosities.⁴⁰ We observed synaptotagmin 1 labeled varicosities around nNOS positive neurons (Figure 4A) and around calretinin positive neurons (Figure 4B). There appeared to be little or no colocalization between synaptotagmin 1 and nNOS varicosities or with calretinin positive varicosities (see Figure 4A and B).

Calretinin labels ascending interneurons, longitudinal motor neurons and intestinofugal neurons.³ In response to local mucosal stimulation, two calretinin positive neurons (N1 and N2) in the following example responded with longer duration bursts of Ca^{2+} transients following each successive stimulation that corresponded with increased activity in synaptotagmin 1 labeled varicosities (Figure 4 B and C and dotted insert). The synaptotagmin 1 labeled varicosities V1, V2, V3 and V4 appeared to be on different nerve fibers since they

had different activities (see below). Furthermore, despite the fact that the first stimulus evoked a CMMC contraction (see y) these neurons were not refractory to subsequent stimuli although a CMMC was not activated.

Effect of blocking N-type Ca^{2+} channels

In 6 preparations, we observed that ω -Conotoxin GVIA (100nM; ω -CTX GVIA), which is a specific blocker of N-type calcium ($\text{CaV}2.2$) channels, substantially reduced Ca^{2+} transients in varicosities (V1–V4) and neurons (N1,N2) during spontaneous activity between CMMCs, see examples in Figure 5A and B. In 3 preparations ω -Conotoxin GVIA caused an increase in the number of spontaneous contractions (Figure 5B). In 3 other preparations, ω -Conotoxin GVIA not only blocked the nerve evoked CMMC response following mucosal stimulation in varicosities (V1–V4) and neurons (N1, N2), but blocked the response in the muscle (Figure 5C, D). Although, ICC-MY continued to exhibit some spontaneous activity in the presence of ω -Conotoxin GVIA (Figure 5D). The increased contractility of the circular muscle (CM) was probably due to the removal of tonic inhibition to the CM and ICC-MY.^{4,5,19,40}

Recruitment of varicosities

Following successive local mucosal stimuli of increasing strength (1 to 3 puffs of N2), there was an increase in the number of active varicosities. An example of this is shown in Figure 6A, where following several local mucosal stimuli the number of active varicosities increased from 126 to ~240.

Another way of analyzing this recruitment of varicose nerve fibers was to threshold the active varicosities before and following stimulation using a bit mask (see Methods). The upper left hand panels in Figure 6B and C show the active varicosities between CMMC and after 8 seconds following a local mucosal stimulus, respectively. The traces in Figure 5D and E shows the % area of varicosities that were actively displaying Ca^{2+} transients, before and following nerve stimulation produced a more sustained firing pattern when compared to between CMMCs (compare All in D and E of Figure 6). It can be observed that a much greater number of varicosities fired during the same 10s period following stimulation (compare Figure 6B and C; and All in D and E). At any time, less than 30% of the total pool of varicosities (All) were active. In this example, 3 individual fibers selected from different parts of the ganglia were thresholded in red; these fibers were active both preceding and 8s following nerve stimulation (see Fibers 1–3 in Figure 6B and C). Their relative area (Figure 6D and E) reflects their degree of activation at a particular time. Individual fibers showed different patterns of activity ranging from constant firing (Fiber 1) or clustered activity (Fiber 3) (Figure 6B and C). In contrast, Fiber 2 rarely fired during the period of stimulation (Figure 6C).

Tracing nerve fibers within the myenteric plexus

By examining the patterns of Ca^{2+} transients in varicosities within myenteric ganglia, we were also able to trace nerve fibers throughout a particular ganglia (See Figure 7A, white insert corresponding to green arrows). In this particular ganglia we were able to trace 19 different nerve fibers by color coding the varicosities and then joining them (Figure 7A and

B). These fibers traced complex, apparently bifurcating paths throughout the ganglia, as well as around and over (and probably under) myenteric neurons (Figure 7B). In a number of cases we were able to trace nerve fibers that appeared to branch several times within a ganglia (Green Fiber 10, Figure 7B). In the following example, Ca^{2+} transients in a varicosity from each of 12 of these 19 fibers, and from two neurons (N1 and N2), before and after local mucosal stimulation are shown in Figure 7C and D, respectively. Before stimulation, 7 of these 19 nerve fibers exhibited little or no activity, whereas, following local mucosal stimulation there was a dramatic increase in activity in varicosities on nerve fibers 1, 3, 10, 12 and 17. The rapid Ca^{2+} transients in nerve fiber 1, which was quiescent before stimulation, lasted for the duration of activity in the two neurons (N1 and N2). In contrast, the varicosity on nerve fiber 5 reduced its activity during the complex, which increased again following the complex (see Figure 7F insert). This nerve fiber was likely part of an inhibitory nerve pathway.^{4,5}

Since a number of axons bifurcated within a ganglia we attempted to determine if there was "branch point failure", i.e. where action potentials in one part of an axon fail to propagate into another branch of the nerve fiber.⁴² To do this we observed the pattern of Ca^{2+} transients in varicosities in different branches of the same nerve fiber over time. We were looking for missing Ca^{2+} transients in the pattern of activity. We examined the activity in 12 branching axons (12 ganglia, n=7) and never found one example of such a phenomena, which is relatively common in other nervous systems (see Figure 7A green arrows and insert).

Correlation of neuronal firing with activity in varicosities

In neurons that displayed ongoing Ca^{2+} transients, we did not find a correlation between this activity with Ca^{2+} transients in neighboring varicosities in approximately 60% of neurons examined (140 neurons, 10 ganglia, n=10) (Figure 8A). When this was the case, these active neurons had so many (8) active varicosities with different activities on or around their soma, as shown in the color coded in the expanded view in Figure 8A (right hand panel, RHP). An example of 4 active varicosities that fired asynchronously in this particular neuron is shown in Figure 8A. By tracing the fibers away from the neuron we could monitor their activity without contamination of activity in the neuron. The activity in the varicosities around this neuron exhibited fairly random activity that occasionally clustered together, but there was no obvious correlation with the spontaneous Ca^{2+} transients in the neuron (N) (Figure 8A).

However, we did find some examples where the activity in an apposed varicosity closely matched the activity in a neuron, suggesting that the neuron was likely activated by neurotransmitter release from the varicosity (Figure 8B, C and D). Two of these neurons exhibited spontaneous activity that was closely matched by Ca^{2+} transients in close varicosities (Figure 8B and C). Figure 8D is an example of evoked rapid Ca^{2+} transients in a neuron that was closely matched by those in the neighboring varicosity.

AH neurons are preferentially stained by mitotracker, and are also calbindin positive.^{4,5} Figure 9A is an example of the activation of a mitotracker positive neuron (N1) and a mitotracker -ve neuron (N2) following local mucosal stimulation. Note that increased

activity in their neighboring varicosities following stimulation was well correlated with the activation of both these neurons.

We also observed activity in nNOS positive neurons, and nNOS negative neurons, that ceased their activity following nerve stimulation,^{4,5,19} an example of which is shown in Figure 9B. In this particular example, both an nNOS positive neuron (N1) and an nNOS –ve neuron within the ganglia reduced their activities during a spontaneous CMMC (see y), by possibly different mechanisms. The reduction of activity in the nNOS positive neuron (N1) appeared to be caused by a temporary increase in activity in the two varicosities V1 (see insert) and V2, which had different activities (see upper 3 ST-Maps). Both these varicosities are likely releasing an inhibitory neurotransmitter. In contrast, the nNOS negative neuron (N2) ceased its activity apparently in response to a reduction in activity in its neighboring varicosity (V3). Since a fiber in an internodal strand also exhibited a similar withdrawal of activity suggests V3 was likely on a nerve fiber originating outside this particular ganglion (not shown).

DISCUSSION

In the present study we have examined, for the first time, the intricate interneuronal communications within the myenteric plexus that occur via activity in axonal varicosities, which are the major sites of neurotransmitter release. We have attempted to characterize the activity in many varicosities both between and during CMMCs and determine whether we could see their activation of myenteric neurons. A compelling aspect of the current study was the fact that in exploring myenteric axonal varicosities, we were largely recording interneuronal nerve traffic.

We have made a number of important observations concerning activity in varicosities within the myenteric plexus that have not been fully appreciated:

1. Ca^{2+} transients in varicosities often occurred in bursts or fired repetitively producing a sustained rise in Ca^{2+} , especially during a CMMC. Its possible that the more sustained increase in Ca^{2+} during the burst activity, especially during a CMMC, is capable of releasing not only more of a fast neurotransmitter but also (peptidergic) cotransmitters.^{1,3}
2. The complex patterns of activity in varicosities can be simplified since varicosities along the same nerve fiber exhibit identical patterns of activity.
3. The frequency of activity in varicosities corresponds with inhibitory activity of the circular muscle between CMMCs, and some fibers that were quiescent, or showed little activity, fire rapidly following mucosal stimulation that initiates the CMMC.
4. Ca^{2+} transients in some varicosities were temporally correlated with activation of myenteric neurons, including AH neurons.
5. Both excitatory and inhibitory varicosities were observed.
6. Some neurons are surrounded by active varicosities but exhibit little activity. This suggests that either the neurotransmitters released by these varicosities are

inhibitory, or that these are weak inputs that require synchronized activity to generate a threshold activation of this particular neuron.³⁹ Furthermore, the presence of a Ca^{2+} transient in a varicosity doesn't necessarily mean that they are releasing neurotransmitter.^{34,37}

7. Synaptotagmin 1 positive varicosities, which produce rapid exocytosis of vesicles activate calretinin positive neurons, although varicosities that are positive for nNOS or calretinin don't appear to colocalize with Synaptotagmin 1, implying that they have different presynaptic Ca^{2+} sensing proteins involved in neurotransmitter release.⁴¹

Ca^{2+} transients in varicosities

Single Ca^{2+} transients in varicosities produced consistent rates of rise and transient durations, suggesting that most unitary Ca^{2+} transients in a varicosity were activated by single action potential propagating from a neuron cell body and down the nerve fiber. This reasoning is supported by the fact that the rate of rise and duration of the unitary Ca^{2+} transients in varicosities was similar, if not identical, to those observed in the varicosities on the terminals of sympathetic neurons following the conduction of a single action potential.^{35–37} This implies that an action potential invading a nerve terminal, which is between 1–3ms in duration, can give rise to a Ca^{2+} transient ~4000 ms in duration by opening mainly ω -Conotoxin (ω CTX) GVIA sensitive N-type Ca^{2+} channels. This amplification is not unusual, since Ca^{2+} influx with a single action potential has been observed in an AH neuron,^{43–45} and following repetitive firing in some S neurons,⁴⁶ which is also largely produced by the opening of N-type Ca^{2+} channels, can give rise to a prolonged (3–4s) Ca^{2+} transient though the release of Ca^{2+} induced Ca^{2+} release (CICR) from intracellular stores.^{43–45} Although CICR doesn't appear to play a major role in the varicosities of sympathetic neurons since their Ca^{2+} transients are insensitive to ryanodine.³⁶

We cannot rule out that ω -Conotoxin (ω CTX) GVIA may have blocked Ca^{2+} influx through voltage gated N-type Ca^{2+} channels on varicosities in particular nerve fibers in interconnected nerve pathways, since presynaptic R-type Ca^{2+} channels also appear to be important for neurotransmitter release in a subpopulation of myenteric neurons.⁴⁸ While it may be argued that blockade of Ca^{2+} transients in varicosities with ω -CTX GVIA was the consequence of blocking activity in neurons, ω -CTX GVIA likely blocks only the Ca^{2+} transient underlying the afterhyperpolarization in neurons, but not their electrical activity.^{46,49}

Tracing nerve fibers

We were surprised to find the sheer number of varicosities that presented Ca^{2+} transients within a myenteric ganglion, between and during CMMCs. Such complexity became manageable as individual nerve fibers containing varicosities were recognized, which had identical activities, allowing us to reduce the various numbers of patterns present. Previous studies have demonstrated that action potentials, propagating down the axon of a single, albeit, sympathetic nerve fiber, produces a unitary Ca^{2+} transient at all varicosities on its terminal axon.¹⁷ We used this observation in our attempts to trace active nerve fibers in the myenteric ganglia, across internodal strands and into interganglionic spaces. Whether

varicosities not directly contacting their target cells were releasing neurotransmitter needs to be explored, since it has been shown that there are a number of different types of synapse, apart from close junctional specializations, that don't make close contacts with target cells but can release neurotransmitters, especially neuropeptides, via volume transmission.⁵⁰

Ca²⁺ transient firing in varicosities between and during CMMCs

In this study, we found that activity in some varicosities could be correlated to Ca²⁺ transients observed in myenteric neurons, although this association was not as tight as might be expected if a strong fiber activated a neuron, as they appear to do on ICC-MY.¹⁹ This is likely due to the fact that activation of most neurons requires a degree of convergence from a number of fibers, whereas, ICC-MY, which are critical for activating the muscle, only appear to be activated by several varicosities on the same motor nerve fiber.¹⁹

Between CMMCs we observed a number of quiescent varicosities; whereas, during the CMMC silent varicosities were activated and displayed high frequency Ca²⁺ transients. Between CMMCs the colon is in a state of tonic inhibition, since inhibitory motor neurons release both nitric oxide and ATP to generate an overall hyperpolarization of the circular muscle and ongoing inhibitory junction potentials (IJs) respectively^{22–24, 27} and a suppression of activity in ICC-MY.¹⁹ Interestingly, the overall frequency of firing in varicosities was found to be 0.6Hz, similar to the frequency of spontaneous IJs.²³ This suggests that many of these nerve fibers are likely to be interneurons in descending inhibitory nerve pathways that activate nNOS positive inhibitory motor neurons.⁵

During the CMMC some varicosities on different nerve fibers synchronized their activity and fired at an average frequency of 1.3Hz (max 2.0Hz) for the duration of the CMMC, similar to what has been described for ascending interneurons,²⁸ and is similar to the duration and firing frequency of the fast Ca²⁺ transients reported in the circular muscle and ICC-MY during the CMMC.^{4,5,24} The increased number of varicosities recruited, as well as their increased frequency of firing, during the CMMC suggests that they lead to the repetitive activation and possibly activate excitatory motor neurons.

Some inhibitory motor neurons reduce their activity during the CMMC, whereas excitatory motor neurons in ascending excitatory nerve pathways release both ACh and tachykinins to cause rapid Ca²⁺ transients superimposed on a sustained increase in Ca²⁺ in ICC-MY and the longitudinal and circular muscle,^{4,22–24,27,19} which correspond to the rapid electrical oscillations that are superimposed on slow depolarization of the muscle.^{23,24,23} Since a number of these varicosities were quiescent prior to stimulation, it is likely that they may be ascending interneurons, some of which may be calretinin positive.³ Such activity is due to the interaction of large numbers of neurons, many of which have axonal fibers that may span across several ganglia.^{2,13,16,49} An interesting finding was that nNOS positive neurons ceased their activity during the CMMC by apparently being directly depressed by release of an inhibitory neurotransmitter from an active varicosity. What this inhibitory neurotransmitter might be is unknown; it is unlikely to be nitric oxide since this reduction in activity is still observed following blockade of nitric oxide synthesis.⁵ This inhibition is likely to be presynaptic inhibition, perhaps by a transmitter released from ascending interneurons.

Potential significance for recording Ca^{2+} transients in intermodal strands

Recordings made across an entire internodal strand revealed complex activity, comprised of many different varicose nerve fibers that together fired at ~1Hz between CMMCs. When traces were differentiated, the resulting traces resembled multiunit electrical recordings of action potentials from complex afferent nerves.⁵¹ Such an approach of examining varicosity activity across multiple fibers could thus be used to examine traffic signaling through interganglionic connectives during motor behaviors, since the single unit Ca^{2+} transients could be analyzed with a suitable thresholding of their amplitude.

In conclusion, this study describes the functional activity of large groups of axonal varicosities in the myenteric plexus of the murine colon, both between and during CMMCs. This overall activity could closely be correlated to activity observed in myenteric neurons, as well as the effector cells that they innervate. The understanding of such synaptic integration within the enteric nervous system is essential in our attempts to unravel the means by which complex neural networks communicate and how they exert their activities in the generation of motor behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tracing varicose axons in internodal strands

A. Left hand panel (LHP): A single frame, inverted to show an active varicose nerve fiber. AI) Ca²⁺ transients taken from a single varicosity along this nerve fiber (see ** arrow, LHP) expressed as a line trace, a differentiated trace and a Spatio-temporal map (ST-Map). Bottom panel shows an inverted imaging of the summed Ca²⁺ activity over 1000 frames (Fluo-4 max, inverted image). AII) Ca²⁺ transients taken from the whole rectangular region of interest (red ROI in LHP), and expressed as an ST-Map below. Green region in ST-Map includes the varicose nerve fiber in I. AIII) Ca²⁺ transients taken from the blue rectangle. B. LHP: Summed activity in the same internodal strands shown in A. AI Ca²⁺ transients taken from 8 selected varicosities (orange traces) throughout the internodal strands that exhibited identical patterns of activity, suggesting they were on the same nerve fiber (see orange arrows in LHP). The Ca²⁺ transients in each varicosity (BI 1–8) were summed together in BI i. BI ii–vi Using the same method Ca²⁺ transients in 8 other varicosities from 6 other nerve fibers were traced within the internodal strands and summed together (BI ii–vi colored traces were sampled at the colored arrows (LHP and BI ii–vi)). BII The entire summed activities of fibers i–vi are overlaid, each Ca²⁺ transient peak is represented as a colored * below.

Figure 2. Ca^{2+} transients in multiple varicosities

A. Inverted LHP shows the summed Ca^{2+} activity in the ganglia, revealing numerous active varicosities (dark spots). RHP shows Ca^{2+} transients from 10 of these varicosities recorded throughout the ganglia and around an ICC-MY in the extraganglionic space. Colored vertical lines below shows the peak of every Ca^{2+} transient. B. Ca^{2+} transients from 8 varicosities lying over neuron N, which only fired once throughout the recording, show different activities. Hexamethonium (100 μM) blocked the activity in most of these particular varicosities. Several local mucosal stimuli led to firing in two varicosities and responses in neurons (N1 and N2). D. Following local mucosal stimulation, varicosities V1 and V2 eventually responded with a prolonged burst of high frequency Ca^{2+} transients. At the same time neurons N1 and N2 also responded. B. Hexamethonium (100 μM ; Hex) blocked the responses in these varicosities, but only inhibited the responses in N1 and N2.

Figure 3.
A plot of the frequency of varicosities both between and following CMMCs.

Figure 4. Responses in synaptotagmin 1 labeled varicosities and calretinin positive neurons
 A. I nNOS labeled neuron. II. Synaptotagmin 1 labeled varicosities. III. Superimposed nNOS with synaptotagmin 1. Synaptotagmin 1 varicosities surround nNOS neurons, but the nNOS varicosities don't appear to be colocalized with synaptotagmin 1 (see arrows). III. Another ganglia showing the lack of colocalization of nNOS and synaptotagmin 1 varicosities. B. LHP shows the lack of colocalization of calretinin positive neurons with synaptotagmin 1 labeled varicosities that surrounded these neurons. C. Ca^{2+} transient responses in several synaptotagmin 1 labeled varicosities V1, V2, V3 and V4 that were closely opposed to two calretinin positive neurons (N1 and N2), respectively. The first local mucosal stimulus elicited a CMMC like response (see contraction Δy). However, despite the refractoriness associated with the CMMC these neurons responded to subsequent stimulation. There was often a close association between the responses in the synaptotagmin 1 labeled varicosities and those in the neurons (see expanded panel in B). (movement artifact shown in red dotted rectangle).

Figure 5. Effect of blocking N-type Ca^{2+} channels

A. Spontaneous activity in varicosities (V1–V4) and neurons (N1, N2). Δy = movement. B. Following ω - Conotoxin GVIA (100nM; ω -CTX GVIA) the majority of the activity was blocked, although there was an increase in spontaneous contractions (Δy). C. Anal stimulation of the mucosa evoked a robust CMMC response. Underlying this CMMC was an intense prolonged burst of Ca^{2+} transients in varicosities V1, V2 and V4, whereas V3 displayed a transient burst of activity. This increase in activity was reflected in the Ca^{2+} transients in neurons N1 and N2, the ICC-MY and the circular muscle (CM). D. Following ω - Conotoxin GVIA (100nM) all the responses to stimulation were blocked; although ICC-MY developed spontaneous activity.

Figure 6. Recruitment of varicosities and tracing nerve fibers

A. Active varicosities (red dots) are shown throughout the ganglia (inverted images) in control and following 3 successive local mucosal stimuli (puffs of N_2). B. The summed activity of all varicosities that display Ca^{2+} transients between CMMCs (see Methods; All; 10s) and 3 individual fibers selected from different parts of the ganglia thresholded in red. Traces of the % area of varicosities that were actively displaying Ca^{2+} transients. At any time, less than 30% of the total pool of varicosities (All) were active. Individual fibers showed different patterns of activity ranging from constant firing (Fiber 1), quiescence (Fiber 2) or clustered activity (Fiber 3). C. The summed activity of all varicosities that display Ca^{2+} transients 8 s after a brush-stroke stimulus (All). Note the much greater number

of varicosities that fired during the same 10s period. The same individual fibers were selected (Fibers 1, 2 & 3). D. shows traces of the % area of varicosities that were actively displaying Ca^{2+} transients. Note the more sustained firing pattern after the brush-stroke compared to between CMMCs. The activity in the individual fibers was altered compared to between CMMCs; activity in Fiber 1 became more irregular and activity in Fibers 2 & 3 were decreased.

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Figure 7. Activity in varicosities between and following the CMMC

A. By color coding varicosities with similar activity (see white insert and corresponding green arrows) 19 nerve fibers were traced throughout the ganglia. B. These varicosities were joined to show the course of the different nerve fibers. Varicosities along each nerve exhibited the same pattern of Ca^{2+} transients. C. and D. Spontaneous and evoked (local mucosal stimulation) Ca^{2+} transients in 12 varicosities selected from 12 of the 19 fibers in A & B, plus the two neurons (N1 and N2). D. Following successive local mucosal stimulation there was a pronounced increase in activity in N1 and N2, and in fibers 1, 2, 3, 4, 10, 12, 17; whereas, the activity in fiber 5 ceased firing during the CMMC response (see F).

Figure 8. Correlation between Ca^{2+} transients in varicosities and neurons

A. This particular neuron (N) exhibited spontaneous activity. 6 different active nerve fibers were traced to this neuron, whose varicose fibers are color coded are shown in the expanded panel (RHP). Despite their ongoing activity there was little correlation between their activity and that in the neuron (N). B and C. Spontaneous activity in these neurons was closely matched by the activity in a closely associated varicosity. D. The second local mucosal stimulus evoked a burst of Ca^{2+} transients in a varicosity that produced a similar burst in the neuron (N).

Figure 9. Excitatory responses in mitotracker positive neuron and inhibitory responses in neurons

A. Each local stimulation of the mucosa produced a transient response in neuron 1 and in neuron 2. Neuron 1 was mitotracker positive, whereas neuron 2 was mitotracker -ve (see lower panel). These responses were preceded by activity in their closely associated varicosities V1 and V2. B. During a spontaneous CMMC (see contraction Δy), both the nNOS positive neuron (N1) and the nNOS -ve neuron (N2) ceased their activity (see corresponding ST-Maps). However, the reduction in activity in the nNOS positive neuron was preceded by an increase in activity in varicosities V1 (see expanded panel) and V2. In contrast, the nNOS -ve neuron (N2) reduced its activity as the activity in varicosity V3 ceased.